Online Supplementary Material

Plasma Metabolomic Profile in Chronic Thromboembolic Pulmonary Hypertension

Methods

Quantitation of Free Fatty Acids Using HPLC Online Tandem Mass Spectrometry (LC/MS/MS)

1. Chemicals and solvents

All the fatty acids (FA) standards from 16 to 22 carbons including the saturated and unsaturated were purchased from Cayman Chemical (Ann Arbor, Michigan).

2. Sample preparation

A 20 µl human plasma sample was mixed with 80 µl of methanol containing 2.5 ug/ml internal standard heneicosapentaenoic acid (FA(21:5); HPA) and vortexed for 30 sec. After centrifuging at 18000 rcf for 10 min, 40 µl of supernatant was transferred into a vial for FA analysis by LC/MS/MS.

3. LC/MS/MS analysis

A triple quadrupole mass spectrometer (Thermo Quantiva) was used for analysis of FA. A volume at 2 µl was injected onto a C18 column (Gemini, 3 µm, 2 x 150mm, Phenomenex) for the separation of FA species. Mobile phases were A (water containing 0.1% acetic acid) and B (methanol/acetonitrile (50/50) containing 0.1% acetic acid and 0.06% ammonium hydroxide). The run started with 75% mobile phase B from 0 to 2 min at the flow rate of 0.3 ml/min. Solvent B was then increased linearly to 100% B from 2 to 8 min and held at 100% B from 8 to 18 min. The column was finally re-equilibrated with 75% B for 8 min. The HPLC eluent was directly injected into the triple quadrupole Thermo Quantiva and the FA species were ionized using electrospray ionization at negative mode. All the fatty acids were analyzed using Selected Reaction Monitoring (SRM) and the SRM transitions (m/z) were their precursor to precursor ions (m/z) as 255 > 255 for FA(16:0), 253 > 253 for FA(16:1), 283 > 283 for (18:0), 279 > 279 for FA(18:2), 277 > 277 for FA(18:3), 275 > 275 for FA(18:4), 311 > 311 for FA(20:0), 309 > 309 for FA(20:1), 307 > 307 for FA(20:2), 305 > 305 for FA(20:3), 303 > 303 for FA(20:4), 301 > 301 for FA(20:5), 339 > 339 for FA(22:0),

333 > 333 for FA(22:3), 331 > 331 for FA(22:4) 329 > 329 for FA(22:5), 327 > 327 for FA(22:6) and 315 > 315 for HPA (internal standard).

4. Data analysis

Peak areas for all the FA species and the internal standards were integrated using the software Xcalibur. Internal standard calibration curves were used for quantitation of FA species in human plasma. We used one way ANOVA to compare FAs levels across the 3 groups, and the Tukey test to compare levels between groups while adjusting for multiple comparisons.

e-Table 1. Statistically significantly different compounds from a total of 862 metabolites measures

	CTEPH / Control	CTEPH / IPAH
Total biochemicals $p \leq 0.05$	362	147
Biochemicals (↑ | ↓)	178 | 184	45 | 102

e-Table 2. Acyl cholines

Biochemical	CTEPH/Control	p value	q value
palmitoylcholine	0.42	1.86E-06	2.04E-05
oleoylcholine	0.43	2.07E-06	2.16E-05
palmitoloelycholine	0.43	6.40E-06	5.69E-05
dihomo-linolenoyl-choline	0.36	5.31E-07	9.11E-06
linoleoylcholine*	0.38	1.76E-06	2.02E-05
stearoylcholine*	0.4	1.57E-06	1.84E-05
docosahexaenoylcholine	0.4	1.22E-07	3.93E-06
arachidonoylcholine	0.45	5.65E-06	5.12E-05
eicosapentaenoylcholine	0.5	2.00E-05	0.0001

Data presented as fold change between CTEPH and control group.

e-Table 3. Associations between selected metabolites and right heart hemodynamics in CTEPH patients. Coefficients and p values for the biochemical calculated by multiple regression modeling adjusting for age, gender, BMI, statin use, thyroid replacement therapy, steroids and diabetes drug therapy.

	Cardiac Index	p	TPR	p
eicosenoate (20:1)	-0.50	0.0007	2.71	0.005
dihomo-linoleate (20:2n6)	-0.41	0.008	1.94	0.05
glycerol	-0.24	0.09	1.09	0.23
acetylcarnitine	-0.41	0.16	1.88	0.30
3-hydroxybutyrylcarnitine	-0.06	0.43	0.55	0.25
3-hydroxybutyrate	-0.03	0.42	0.34	0.09
pimelate	-0.11	0.64	-0.62	0.66
palmitoylcholine	0.36	0.05	-1.56	0.19
oleoylcholine	0.35	0.02	-1.39	0.15
1-palmitoyl-GPA (16:0)	-0.14	0.72	-0.74	0.75
2-stearoyl-GPE (18:0)	0.28	0.52	-2.53	0.36
ornithine	0.04	0.95	-4.73	0.34

BMI, body mass index; GPA, glycerol-3-phosphate; GPE, glycerophosphoethanolamine; TPR, total pulmonary vascular resistance.

e-Table 4. Plasma fatty acids measured by high performance liquid chromatography (HPLC) Online Tandem Mass Spectrometry (LC/MS/MS).

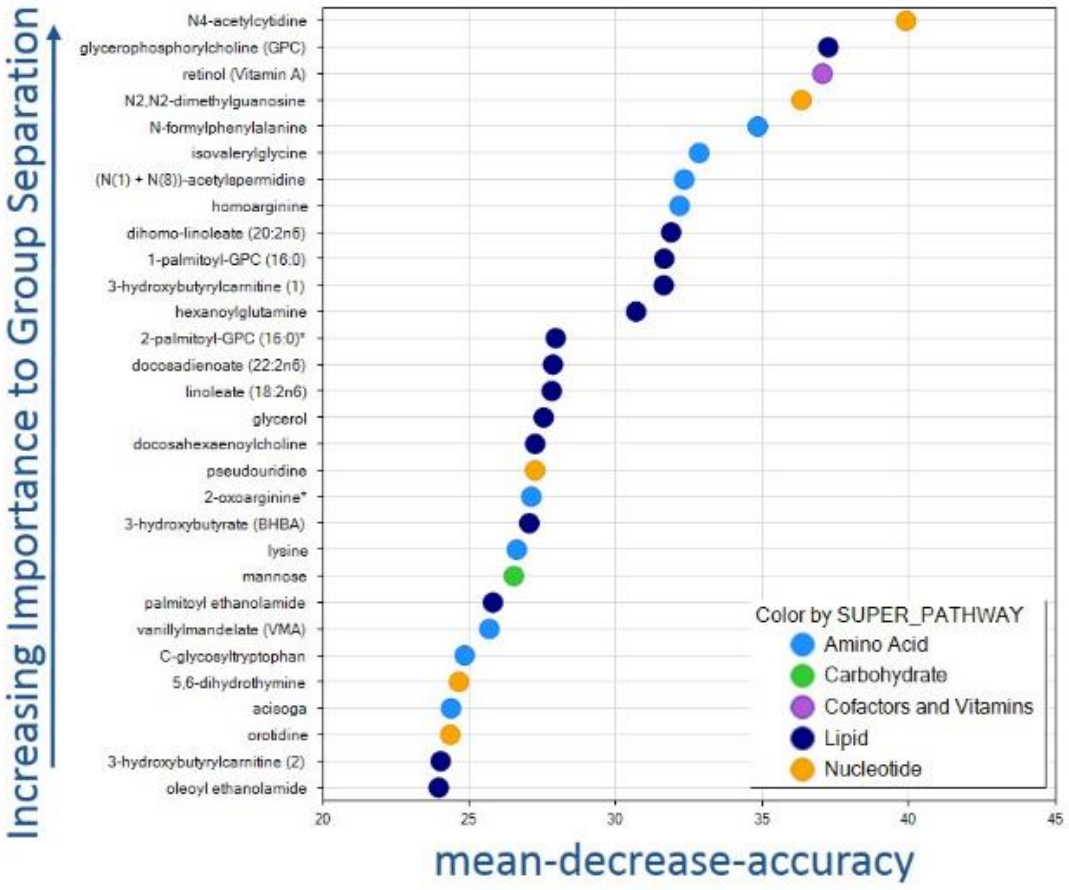
	Control N = 31	CTEPH N = 33	IPAH N = 21	p
Palmitate (16:0)	172.19 (91.00)	304.27 (144.89)	191.81 (90.52)	<0.001
Palmitolate (16:1)	18.21 (13.77)	47.57 (32.64)	32.80 (26.76)	<0.001
Stearate (18:0)	48.11 (26.21)	77.42 (32.24)	59.00 (24.28)	<0.001
Oleate (18:1)	164.46 (114.97)	393.19 (195.99)	247.65 (146.59)	<0.001
Linoleate (18:2)	104.64 (60.55)	241.79 (139.69)	143.76 (92.41)	<0.001
Gamma-Linolenate (18:3)	3.02 (2.04)	6.83 (5.33)	3.42 (2.41)	<0.001
Stearidonate (18:4)	0.05 (0.06)	0.08 (0.12)	0.03 (0.02)	0.075
Eicosanoate (20:0)	0.57 (0.44)	0.73 (0.54)	0.53 (0.22)	0.189
Eicosenoate (20:1)	2.46 (1.93)	5.81 (2.99)	3.62 (2.28)	<0.001
Dihomo-linoleate (20:2)	0.57 (0.37)	1.35 (0.69)	0.79 (0.45)	<0.001
Dihomo-gamma-linoleate (20:3)	0.63 (0.26)	1.27 (0.71)	0.73 (0.43)	<0.001
Arachidonate (20:4)	4.25 (1.94)	8.60 (4.94)	5.32 (2.57)	<0.001
Eicosapentaenoate (20:5)	0.47 (0.48)	0.72 (0.76)	0.36 (0.38)	0.076
Docosanoate (22:0)	0.23 (0.23)	0.28 (0.25)	0.18 (0.11)	0.267
Docosatrienoate (22:3)	0.02 (0.01)	0.03 (0.01)	0.02 (0.01)	<0.001
Docosatetraenoate (22:4)	0.65 (0.46)	2.02 (1.23)	1.05 (0.67)	<0.001
Docosapentaenoate (22:5)	0.79 (0.57)	2.06 (1.64)	1.09 (1.00)	<0.001
Docosahexaenoate (22:6)	2.55 (2.17)	3.87 (3.46)	2.07 (1.79)	0.038

All concentrations in micromolar (uM).

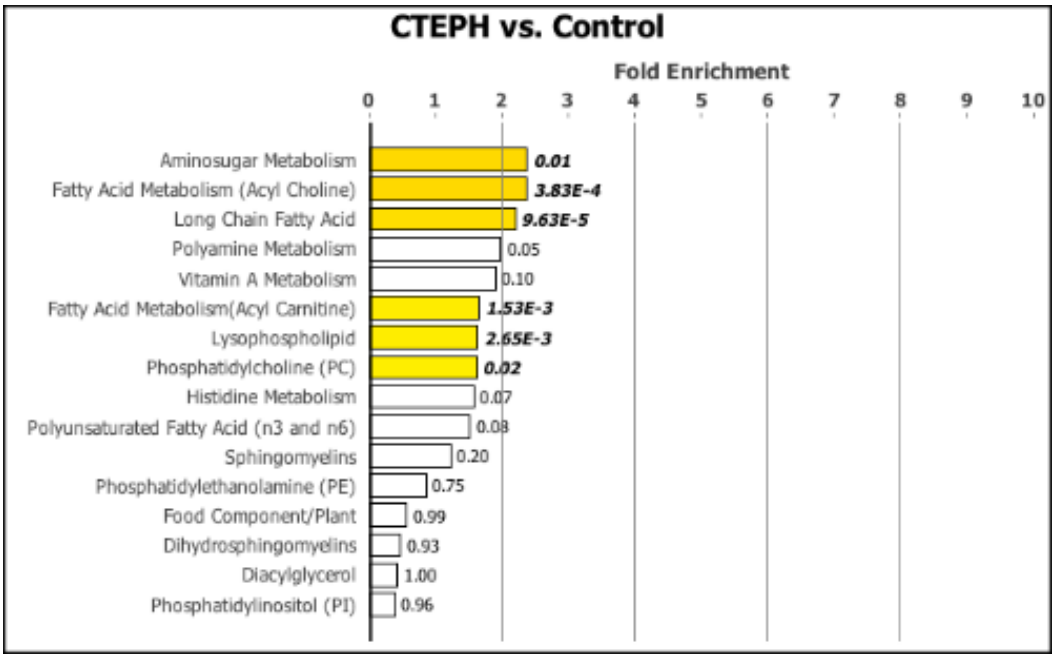
Data are presented as mean (standard deviation)

Figures

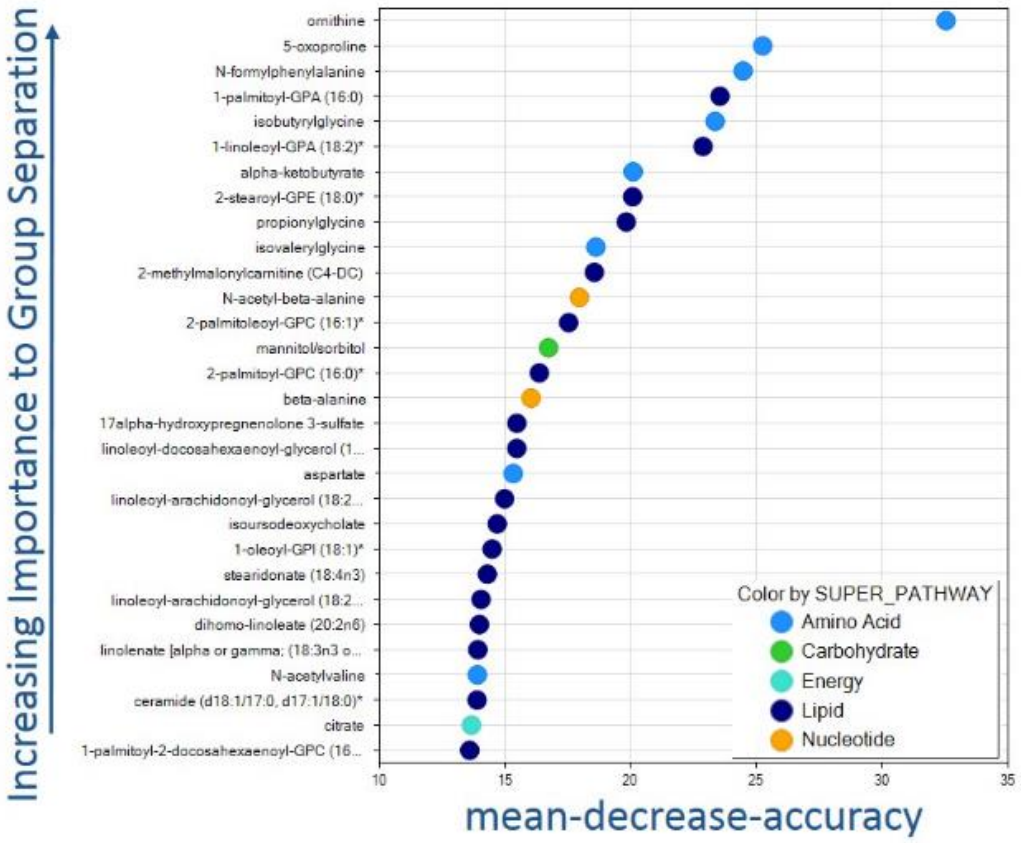
e-Figure 1. Random Forest classification using named metabolites comparing plasma samples from subjects with CTEPH and healthy controls gave a predictive accuracy of 89%. The 30 top ranking metabolites in the importance plot suggest key differences in nucleotides, lipids and amino acids.



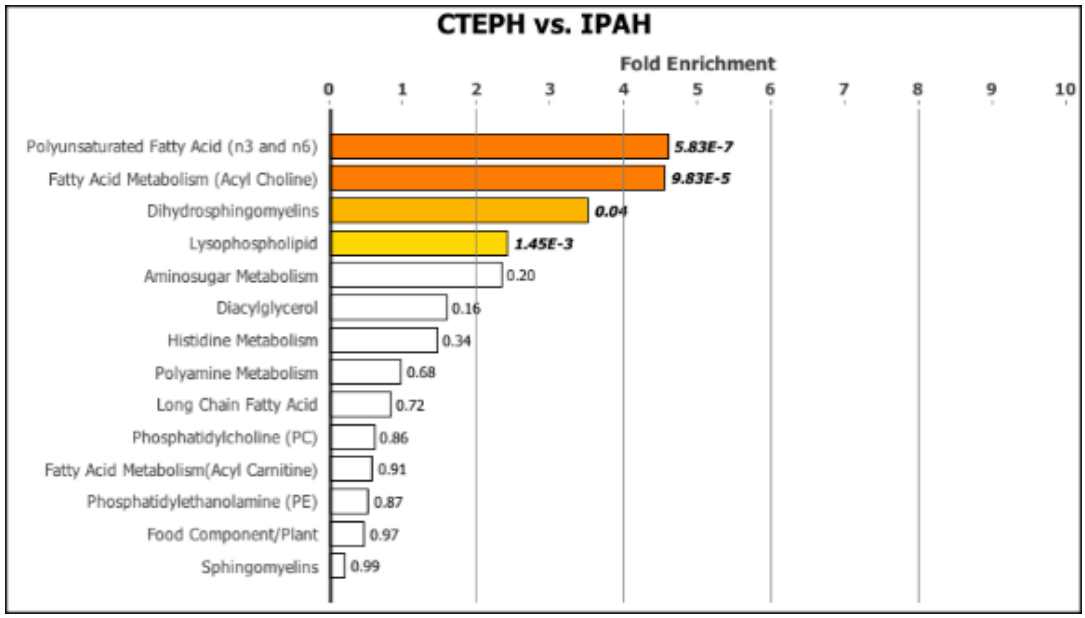
e-Figure 2. Pathway enrichment analysis shows that amino sugar metabolism, acyl choline, long chain fatty acid (LCFA), acyl carnitine, lysophospholipid and phosphatidylcholine pathways are enriched in CTEPH patients compared to controls. Numbers next to bars represent p values.



e-Figure 3. Random Forest classification using named metabolites comparing plasma samples from subjects with CTEPH and IPAH gave a predictive accuracy of 80%. The 30 top ranking metabolites in the importance plot suggest key differences in lipids and amino acids.



e-Figure 4. Pathway enrichment analysis identified differences in polyunsaturated fatty acids, acyl cholines and lysophospholipids between CTEPH and IPAH patients. Numbers next to bars represent p values.



e-Figure 5. Changes in fatty acids and glycerol after pulmonary endarterectomy in 5 CTEPH patients.